Preference of DNA Methyltransferases for CpG Islands in Mouse Embryonic Stem Cells

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Many CpG islands have tissue-dependent and differentially methylated regions (T-DMRs) in normal cells and tissues. To elucidate how DNA methyltransferases (Dnmts) participate in methylation of the genomic components, we investigated the genome-wide DNA methylation pattern of the T-DMRs with *Dnmt1*-, *Dnmt3a*-, and/or *Dnmt3b*-deficient ES cells by restriction landmark genomic scanning (RLGS). Approximately 1300 spots were detected in wild-type ES cells. In *Dnmt1*$^{-/-}$ ES cells, additional 236 spots emerged, indicating that the corresponding loci are methylated by Dnmt1 in wild-type ES cells. Intriguingly, in *Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$ ES cells, the same 236 spots also emerged, and no additional spots appeared differentially. Therefore, Dnmt1 and Dnmt3a/3b share targets in CpG islands. Cloning and virtual image RLGS revealed that 81% of the RLGS spots were associated with genes, and 62% of the loci were in CpG islands. By contrast to the previous reports that demethylation at repeated sequences was severe in *Dnmt1*$^{-/-}$ cells compared with *Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$ cells, a complete loss of methylation was observed at RLGS loci in *Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$ cells, whereas methylation levels only decreased to 16% to 48% in the *Dnmt1*$^{-/-}$ cells. We concluded that there are CpG islands with T-DMR as targets shared by Dnmt1 and Dnmt3a/3b and that each Dnmt has target preferences depending on the genomic components.

It is estimated that the human and mouse genomes consist of ~3 × 10^9 bp/haploid genome but contain only 30,000 to 40,000 genes. A large amount of the genome is composed of nongenic repetitive elements (~41% and 48% in human and mouse, respectively), including interspersed repeats and satellites (Lander et al. 2001; Venter et al. 2001; Waterston et al. 2002). Most CpGs are methylated in the mammalian genome; the value varies between 60% and 90% depending on the report (Gruenbaum et al. 1981; Razin et al. 1984; Cross and Bird 1995). It is likely that overall levels of DNA methylation reflect the hypermethylation status of nongenic repeated sequences rather than gene encoding areas.

CpG islands are genomic regions with high GC content and frequent CpG appearance compared with the entire genome (Gardiner-Garden and Frommer 1987) and are often located in promoter regions in the 5′ flanking region of housekeeping genes and many tissue-specific genes (Bird 1987; Gardiner-Garden and Frommer 1987; Larsen et al. 1992). The numbers of housekeeping genes and tissue-specific genes in the human and mouse were calculated by Antequera and Bird (1993). The human and mouse genome projects identified ~15,500 and 29,000 CpG islands in the mouse and human genomes, respectively (Lander et al. 2001; Venter et al. 2001; Waterston et al. 2002), and a recent database analysis suggested that approximately half of the tissue-specific promoters are linked with CpG islands (Suzuki et al. 2001). Although most CpG dinucleotides in the mammalian genome have been found to be methylated at the cytosine residue (Cooper and Krawczak 1989), CpG islands were once considered to be unmethylated regions in normal tissue. However, there are numerous tissue-dependent and differentially methylated regions (T-DMRs) in CpG islands in normal cells or tissues, and the methylation profile of the CpG islands is unique in each tissue or cell type (Shiota et al. 2002).

Information is still limited concerning DNA methylation in gene coding regions, although aberrant DNA methylation in these regions is known to occur in cancers (Jones and Baylin 2002). It is still obscure how DNA methylation, which is regulated by DNA methyltransferases (Dnmts), occurs at T-DMRs of CpG islands. To date, three classes of Dnmts—Dnmt1, Dnmt2, and Dnmt3—have been identified in mammals. Methyltransferases Dnmt1 and Dnmt3a/3b are found to be functional both in vitro and in vivo. In vitro studies indicated that Dnmt1 prefers hemimethylated DNA compared to unmethylated DNA (Bestor 1992; Yoder et al. 1997; Pradhan et al. 1999), whereas Dnmt3a/3b methylate CpG dinucleotides without preference for hemimethylated or unmethylated DNA (Okano et al. 1998). Collectively, the fact that Dnmt1 is localized to DNA replication foci (Leonhardt et al. 1992) and is associated with MeCP2, which directs DNA methyltransferase activity to hemimethylated DNA (Kimura and Shiota 2003), implies that Dnmt1 is involved in maintenance methylation in vivo to preserve methylation patterns in genomic DNA, and that Dnmt3a/3b function as de novo methyltransferases. Studies using *Dnmt1*$^{-/-}$ and *Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$ ES cells demonstrated that demethylation occurred at repeated sequences, such as endogenous C-type retroviruses, more extensively in *Dnmt1*$^{-/-}$ cells than in *Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$ cells, and that some imprinted genes such as *Igf2r* and *H19* became demethylated only in *Dnmt1*$^{-/-}$ cells but not in *Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$ cells (Okano et al. 1999). Recently, a direct association between DNMTs was reported (Kim et al. 2002), and it was demonstrated that DNMT1 and DNMT3B function cooperatively for maintenance methylation in the hu-

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man cancer cell line (Rhee et al. 2002). Therefore, categorizing Dnmts into maintenance and de novo DNA methylation may not be appropriate when attempting to characterize in vivo mechanisms involved in determining or establishing DNA methylation profiles in the genome.

Dnmts are of critical importance for development (Li et al. 1992; Okano et al. 1999). Formation of DNA methylation profiles underlies mammalian development (Shiota and Yanagimachi 2002), and abnormal methylation status induces various abnormal phenotypes (Ohgane et al. 2001). Systematic survey of DNA methylation patterns regarding CpG islands is one of the most interesting ways to study DNA methylation from the aspect of epigenetic participation in the differentiation and development of mammals. To address the question how DNA methylation in the T-DMR of CpG islands is regulated by Dnmts in vivo, the genome-wide DNA methylation in *Dnmt1-*, *Dnmt3a-*, and/or *Dnmt3b*-deficient ES cells was assessed by restriction landmark genomic scanning (RLGS). We used NotI as a landmark enzyme to focus on CpG islands, because >90% of NotI recognition sites are located in CpG islands (Lindsay and Bird 1987).

RESULTS

Genome-Wide Demethylation of Gene Areas Associated With CpG Islands in Dnmt-Deficient ES Cells

In the RLGS profile, the spot is visible if the corresponding NotI site in the genome is unmethylated or hypomethylated, whereas it is invisible if the site is hypermethylated. We have observed that the methylation status of the NotI site reflects those of the surrounding CpGs in several cases (Imamura et al. 2001; Ohgane et al. 2001). There were ~1300 spots when wild-type ES cells were assessed (data not shown), suggesting that these 1300 genomic sites are hypomethylated in ES cells despite the expression of all types of Dnmts (Chen et al. 2003). Considering our previous observation that there are both methylated and unmethylated loci in cells and tissues and that the methylation pattern at such loci is unique depending on the cell type (Shiota et al. 2002), there should be methylated and undetectable loci in addition to the unmethylated 1300 loci in wild-type ES cells. If so, Dnmts responsible for DNA methylation at NotI sites, probably in CpG islands, can be identified by comparing the RLGS profile of wild-type cells with those of Dnmt-deficient cells.

Two hundred thirty-six spots emerged in the RLGS profile of *Dnmt1*$^{-/-}$ ES cells in addition to the basal 1300 spots in wild-type cells (Fig. 1A). This implies that there are many loci that are methylated by Dnmt1 in ES cells, considering the fact that ≥15,000 CpG islands exist in the mouse genome (Waterston et al. 2002). The intensities of these emerging spots in *Dnmt1*$^{-/-}$ cells were considerably less than those of the unchanged authentic spots (Fig. 1B,C). This suggests that demethylation of T-DMR caused by Dnmt1 deficiency was not complete. Intriguingly, the identical 236 spots found in *Dnmt1*$^{-/-}$ ES cells also emerged in the RLGS profile of the double-mutant (*Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$) ES cells, suggesting that all spots emerging in the RLGS profiles of *Dnmt1*$^{-/-}$ and *Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$ ES cells should be common targets of

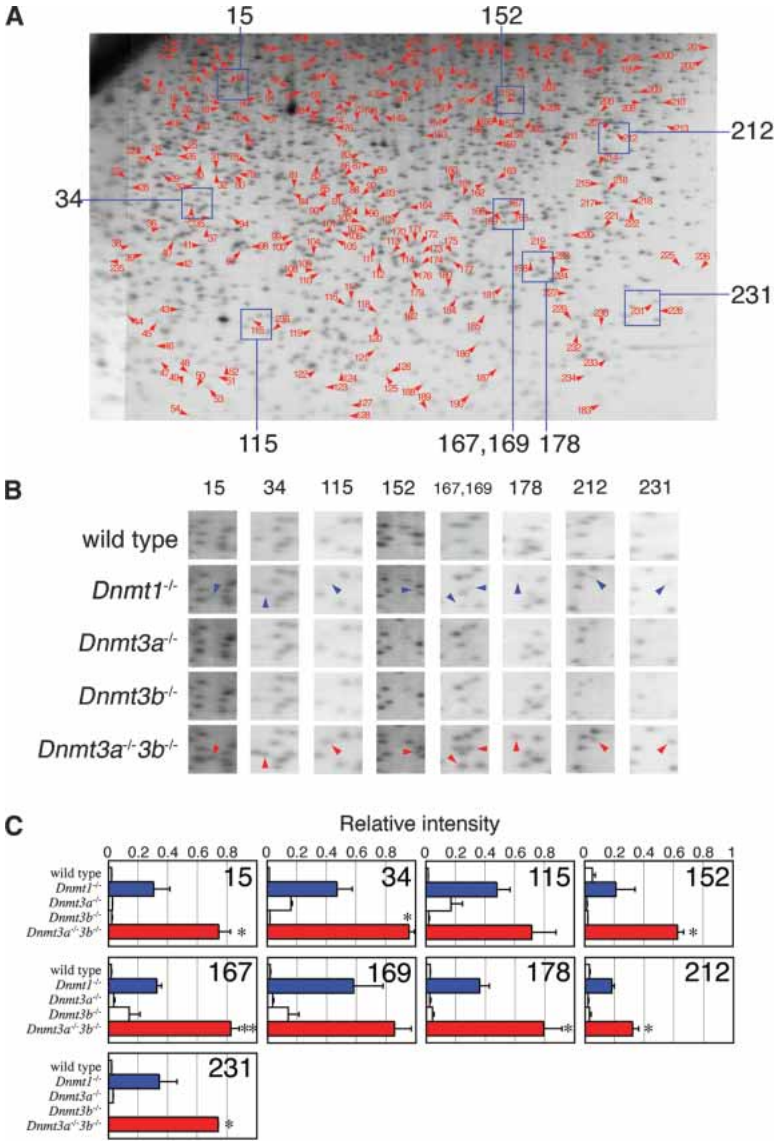


Figure 1 DNA methylation status of NotI sites spread throughout the genome in wild-type and Dnmt-deficient ES cells. (*A*) RLGS profile of *Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$ ES cells is shown as a representative; 236 spots indicated by arrowhead with number were invisible in the wild-type, *Dnmt3a*$^{-/-}$, and *Dnmt3b*$^{-/-}$ single-mutant ES cells but emerged in *Dnmt1*$^{-/-}$ and *Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$ ES cells. Corresponding genomic loci of these spots were hypomethylated by the loss of Dnmt1 or Dnmt3a/3b. (*B*) Enlarged RLGS spots of wild-type and Dnmt-deficient ES cells. Areas with indicated numbers are represented in *A*. Spots with arrowhead exist in the profiles in *Dnmt1*$^{-/-}$ and *Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$ ES cells but are invisible in those of wild-type, *Dnmt3a*$^{-/-}$, and *Dnmt3b*$^{-/-}$ ES cells. Note that intensity of all spots in *Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$ ES cells (red arrowheads) is greater than those in *Dnmt1*$^{-/-}$ (blue arrowheads). (*C*) Relative intensities of the representative RLGS spots. The intensity of each spot was digitized and averaged from three independent RLGS profiles and normalized by the average intensity of the surrounding invariant spots. Differences between *Dnmt1*$^{-/-}$ and *Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$ ES cells were compared by *t* test. **P* < 0.1 ***P* < 0.01 (n = 3)

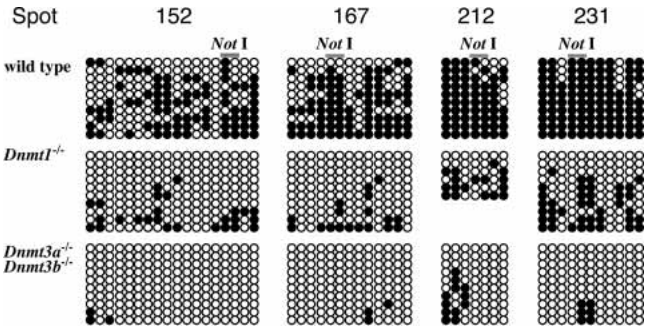


Figure 2 DNA methylation status at CpG sites around the NotI sites. Methylation status around ~600-bp genomic region, including the NotI site, was assessed by sodium bisulfite sequencing method. Open circles and closed circles represent unmethylated and methylated cytosine residues, respectively. In wild-type ES cells, all genomic regions are hypermethylated, including the NotI sites, indicating that DNA methylation is maintained at these regions in the wild-type ES cells. In contrast, CpG sites in the same regions are demethylated in *Dnmt1*$^{-/-}$ and *Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$ ES cells. Although the demethylation is moderate in *Dnmt1*$^{-/-}$ ES cells, almost all CpG sites are totally demethylated in *Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$ ES cells, indicating that RLGS analysis reflects DNA methylation status not only at the NotI site but surrounding CpG dinucleotides of the NotI site. These data suggest that Dnmt3a/3b are more significant for DNA methylation in CpG islands than Dnmt1.

both Dnmt1 and Dnmt3a/3b. There is no RLGS spot that differentially appeared or disappeared between *Dnmt1*$^{-/-}$ and *Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$ cells. Therefore, DNA methylation targets were completely overlapped and shared by Dnmt1 and Dnmt3a/3b. However, there might be some spots that differentially increased their intensities in *Dnmt1*$^{-/-}$ and *Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$ cells. It is also likely that some spots were visible in the wild-type ES cells but increased their spot intensities in *Dnmt1*$^{-/-}$ and *Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$ cells. More importantly, the intensities of the 236 spots were greater in *Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$ ES cells than *Dnmt1*$^{-/-}$ cells (Fig. 1C). These results suggest that the levels of demethylation are more extensive in *Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$ cells than in *Dnmt1*$^{-/-}$ cells.

Each single mutant (*Dnmt3a*$^{-/-}$ or *Dnmt3b*$^{-/-}$) ES cell line showed a similar RLGS profile to that of wild-type cells; thus the deficiency of Dnmt3a or Dnmt3b alone seemed to result in no change in DNA methylation at CpG islands (Fig. 1B,C). Therefore, the roles of Dnmt3a and Dnmt3b appear to be redundant, and a compensatory mechanism between Dnmt3a and Dnmt3b exists regarding DNA methylation at least at the genomic sites corresponding to these 236 spots.

Loss of Methylation at the Flanking CpGs of NotI Sites Was Severe in the Double Mutant of Dnmt3a and Dnmt3b Compared With That in DnmtI Mutant Cells

We further investigated the methylation status of the flanking regions up to −600 bp, including the NotI sites by sodium bisulfite genomic sequencing at four genomic loci (Fig. 2). There are 18, 13, seven, and 11 CpGs within 588-, 504-, 490-, and 486-bp genomic regions corresponding to spots 152, 167, 212, and 231, respectively. In the wild-type ES cells, the NotI sites in all regions were almost completely methylated as seen in the RLGS experiment, and overall, the regions were hypermethylated. Methylation levels at the NotI sites vary 30% to 60% in *Dnmt1*$^{-/-}$ ES cells, whereas they are 0% to 30% in *Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$ cells. Furthermore, demethylation in *Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$ ES cells was more extensive than *Dnmt1*$^{-/-}$ cells in the flanking CpGs of the NotI site. These results support the notion that DNA methylation in the regions, including the NotI site, depends on Dnmt3a/3b more significantly than Dnmt1. In the present study, however, we used *Dnmt1*$^{-/-}$ ES cells collected after 36 passages in culture, whereas the mutant cell lines of *Dnmt3a*$^{-/-}$, *Dnmt3b*$^{-/-}$, and *Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$ were collected after 16, 19, and 19 passages, respectively. Taking the fact that culturing time could affect DNA methylation levels, it is also possible that the lower demethylation levels observed in *Dnmt1*$^{-/-}$ ES cells might be due to remethylation activity of Dnmt3a and/or Dnmt3b, which are still present and active in *Dnmt1*$^{-/-}$ ES cells.

Identification of the Target for DNA Methylation in CpG Island

To identify the sequence information for the methylation target in CpG islands, we applied virtual image RLGS (Vi-RLGS), which is the recently developed in silico method for analyzing database sequences (Matsuyama et al. 2003). By matching the RLGS profile with the Vi-RLGS image, we selected the candidate loci for the RLGS spots. Of the total 236 candidate spots, ~54.2% of the candidate spots are in CpG islands, and ~89.4% of the spots are in the gene coding region (Table 1).

We initially confirmed four spots (spot 12, 15, 18, and 235), which are demethylated in *Dnmt1*$^{-/-}$ and *Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$ ES cells by spot cloning with the electroelution method. To confirm the methylation status of more candidate genomic loci in Vi-RLGS image, we used the method based on the methylation-sensitive restriction digestion and quantitative PCR. PCR amplification of genomic DNA following NotI digestion was dependent on the methylation levels revealed by RLGS (Fig. 3A). Methylation levels at selected loci in *Dnmt1*$^{-/-}$

Table 1. Summary of 236 Loci Predicted by Vi-RLGS

	Genes			Nongenic sequences			
	Function known[a]	Function unknown[b]	Predicted[c]	Repeat sequences[d]	Nonrepeat sequences	Total	ND[e]
CpG island	69 (29.2%)	46 (19.5%)	8 (3.4%)	2 (0.8%)	3 (1.3%)	128 (54.2%)	
Non-CpG island	37 (15.7%)	24 (10.2%)	27 (11.4%)	11 (4.7%)	6 (2.5%)	105 (44.5%)	
Total		211 (89.4%)		22 (9.3%)			3 (1.3%)

[a]Genes that encode proteins with known functions.
[b]Including predicted genes supported by mRNA expression and/or ESTs.
[c]Genes predicted by computer software such as Genescan.
[d]Sequences including LINEs, SINEs, LTR elements or DNA elements.
[e]Not determined.

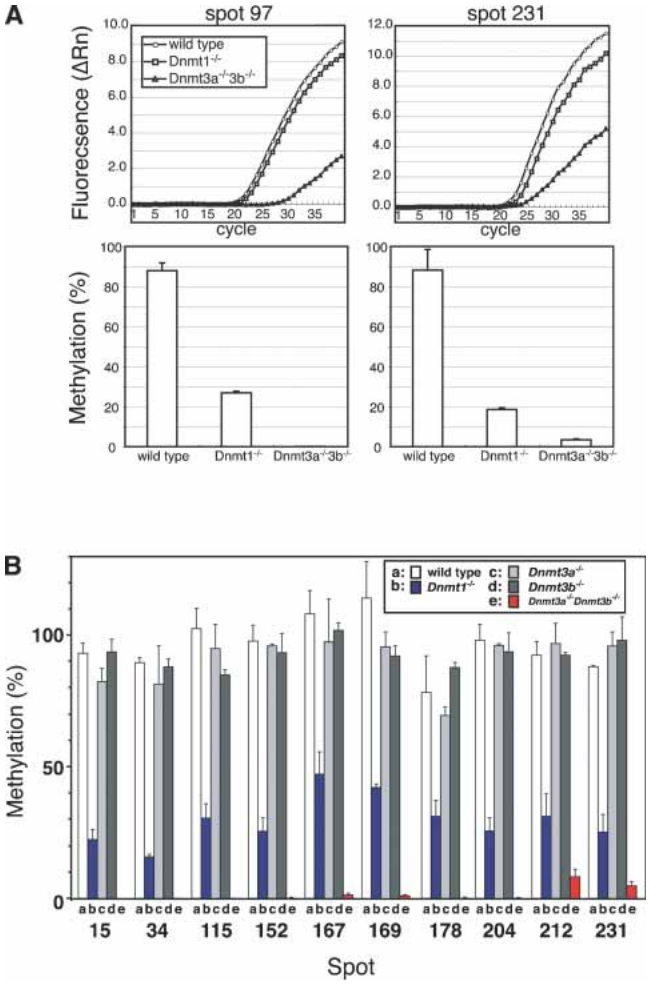


Figure 3 Evaluation of DNA methylation by methylation-sensitive quantitative PCR. (*A*) The amount of undigested genomic DNA after NotI treatment was estimated by real-time PCR using genomic DNA of wild-type, *Dnmt1*$^{-/-}$, and *Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$ ES cells. Spots 97 and 231 are chosen as examples. (*Top* panels) Amplification plots of PCR products from each NotI-treated genomic DNA sample. (*Bottom* panels) Methylation levels at each corresponding genomic site, which are calculated from the ratio of amounts of NotI treated/untreated genomic DNA. For details, see Methods. (*B*) Evaluation of methylation levels at the corresponding genomic loci represented in Fig. 1B in the wild-type, *Dnmt1*$^{-/-}$, *Dnmt3a*$^{-/-}$, *Dnmt3b*$^{-/-}$, and *Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$ ES cells. In the wild-type, *Dnmt3a*$^{-/-}$, and *Dnmt3b*$^{-/-}$ ES cells, all NotI sites were hypermethylated and amplified by real-time PCR at the same levels as NotI-untreated genomic DNA. In contrast, NotI sites were only methylated from 16.1% to 47.5% in *Dnmt1*$^{-/-}$ ES cells and from 0% to 8.5% in *Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$ ES cells.

ES cells were estimated as 16.1% to 47.5% (average, 41.4 ± 3.2%) and those in *Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$ ES cells were evaluated as 0% to 8.5% (average, 5.3 ± 1.0%), whereas the methylation levels in the wild-type, *Dnmt3a*$^{-/-}$, and *Dnmt3b*$^{-/-}$ ES cells were ~100% (Fig. 3B). These data indicate that the genomic locus of each spot can be identified with the combination of Vi-RLGS and the quantitative PCR. We identified 40 genomic loci, including the above four that are methylated in the wild-type ES cells but demethylated in *Dnmt1*$^{-/-}$ or *Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$ ES cells (Table 2). Among these identified loci, 59.0% (23/39) are located in CpG islands, and more importantly, 84.6% (33/39) are in the gene coding regions. Thus, we confirmed that most of the NotI sites reside in the gene coding regions. As far as data obtained in the present study, no imprinted gene was found in Dnmt-deficient ES cells, probably because we focused on spots that did not exist in the wild-type ES cells but emerged in Dnmt-deficient cells.

DISCUSSION

Repetitive elements, including interspersed repeats such as endogenous retroviruses, occupy a large part of the mammalian genome, whereas the area for protein coding genes is very limited (Lander et al. 2001; Venter et al. 2001; Waterston et al. 2002). CpG islands were considered to be clusters of unmethylated CpG dinucleotides except for those in imprinted genes, X-chromosome inactivation, and aberrant methylation in cancer (Bird and Wolffe 1999; Jones and Takai 2001; Reik 2001). There are, however, several CpG island–associated genes that are regulated by DNA methylation in normal tissues (Imamura et al. 2001; Newell-Price et al. 2001; Pao et al. 2001; Futscher et al. 2002; Strichman-Almashanu et al. 2002). In the present study, there were 236 emerging RLGS spots in *Dnmt1*- or *Dnmt3a/3b*-deficient cells in addition to the basal 1300 unmethylated or hypomethylated spots. Cloning of the 236 spots confirmed that they are primarily in the gene encoding regions, and not repeated sequences. In addition to the authentic 247 T-DMRs that are differentially methylated in tissues and cells (Shiota et al. 2002), there are at least 236 T-DMRs that are methylated by Dnmts in ES cells as described below. These T-DMRs are methylated in ES cells but may be unmethylated in other types of cells that we have not explored. The number of T-DMRs in normal cells would be 483, if duplication were not taken into account.

It has been widely accepted that Dnmt1 functions solely as the maintenance DNA methyltransferase, whereas Dnmt3a/3b are involved in de novo methylation. So far, however, maintenance activity by Dnmt1 has been investigated predominantly in repetitive elements such as centromeric/pericentromeric regions as well as broad intergenic regions containing various repetitive elements (Li et al. 1992; Okano et al. 1999). We demonstrated here that Dnmt3a or Dnmt3b is required for DNA methylation in T-DMR of CpG islands. This indicates that Dnmt3a/3b have importance both in maintenance and de novo methylation in CpG islands, at least in ES cells. *Dnmt1* deficiency caused total loss of methylation at the endogenous repeated sequences (Li et al. 1992; Okano et al. 1999). The apparent contribution of Dnmt1 was incomplete and appeared to be less than that of Dnmt3a/Dnmt3b, although Dnmt1 was also involved in DNA methylation in the T-DMRs of CpG islands. Alternatively, both Dnmt1 and Dnmt3a/3b might be necessary and equally contribute to the maintenance of DNA methylation in CpG islands, whereas Dnmt3a and Dnmt3b also had the function of de novo DNA methyltransferases in the same regions. In comparing the overall DNA methylation functions of Dnmt1 and Dnmt3a/3b, Dnmt1 functions as a maintenance methyltransferase both in repeated sequences and CpG islands, whereas Dnmt3 functions more importantly in CpG islands than in other genomic regions (Fig. 4).

Chen et al. (2003) reported that *Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$ ES cells showed loss of DNA methylation in various repeated sequences and some single nonimprinted and imprinted genes. Kinetic analysis indicated that demethylation at repeated sequences in *Dnmt1*$^{-/-}$ ES cells was more rapid than that in *Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$ ES cells, and that demethylation in *Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$ ES cells was progressive and thus exhibited extensive demethylation at late stages of cell culture. They proposed a model for the distinctive roles of Dnmt1 and Dnmt3a/3b, in which Dnmt3 functions as a proofreader to complement the maintenance activity of Dnmt1. In contrast, in

Table 2. Target Loci of Dnmts Detected in RLGS Confirmed by Vi-RLGS and the Methylation-Sensitive Real-Time PCR Method

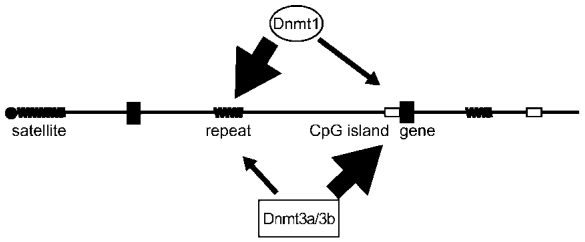
Spot no.[a]	Locus	Gene symbol[b]	Gene name	CpG island
12	15B2	repeat (LTR element)	—	yes
15	5G2	D130017N08Rik	RIKEN cDNA D130017N08 gene	yes
17	11E2	Cbx4	chromobox homolog 4	no
18	8B1.1	—	—	no
21	12D1	LOC238316	predicted gene	yes
25	10B4	4732470K04	predicted gene	yes
30	13A5	LOC238623	predicted gene	yes
31	7B3	Dbx1	developing brain homeobox 1	yes
32	9A5.1	D130038B21Rik	RIKEN cDNA D130038B21 gene	yes
34	18C	AA987150	predicted gene	yes
43	2H3	LOC329560	predicted gene	yes
47	19C3	Trim8	tripartite motif protein 8	yes
53	5B1	BC037112	gene with protein product	no
73	9A4	D730048I06Rik	RIKEN cDNA D730048I06 gene	yes
76	2H3	—	—	no
77	11A4	AL732390.10.1.195612.21298.172306	predicted gene	no
79	10C1	Agpat3	1-acylglycerol-3-phosphate O-acyltransferase 3	yes
87	14D1	Baiap1	BAI1-associated protein 1	no
88	10B4	Mm.338763 (unigene)	—	no
93	7D1	Bnc	basonuclin	yes
95	14C1	MGC38922	predicted gene	yes
97	7A3	Rps19	ribosomal protein S19	yes
99	17E3	Cox7a21	cytochrome c oxidase subunit VIIa polypeptide 2-like	yes
109	13A5	—	—	yes
111	7F3	hmm37743	predicted gene	no
115	7A2	LOC381956	predicted gene	yes
144	13B1	Tgfbi	transforming growth factor, beta induced	no
152	19C3	Pik3ap1	phosphoinositide-3-kinase adaptor protein 1	yes
161	5B2	Man2b2	mannosidase 2, alpha B2	yes
166	5F	Cit	citron	no
167	4D3	LOC230868	predicted gene	no
173	11A4	Odz2	odd Oz/ten-m homolog 2	no
178	2H1	Bfzb-pending	basic FGF repressed, zinc binding protein	no
212	14D2	repeat (DNA element)	—	yes
225	11B1.3	A830006N08	predicted gene	yes
231	16C2	—	—	yes
234	11B5	—	—	no
235	8E2	—	—	no
236	10C1	Pcnt2	pericentrin 2	no

[a]Spot numbers indicate the position in RLGS profile shown in Fig. 1A.
[b]Symbols are shown if the NotI site of the fragment is located within 10 kb of the gene.

the component of the present study focusing on T-DMR of CpG islands, the complete loss of DNA methylation in *Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$ ES cells was observed even at early stages of cell passage, a time when demethylation in the repeated sequences did not progress extensively. This result indicates that *Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$ ES cells lost both activities of de novo and maintenance DNA methylation in CpG islands. The proofreading functions of Dnmt3a/3b may also be different depending on genomic area.

In contrast to *Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$ ES cells, *Dnmt1*$^{-/-}$ ES cells had partially demethylated T-DMRs of CpG islands, although the repeated sequences were fully demethylated in *Dnmt1*$^{-/-}$ ES cells. DNA methylation in CpG islands and gene encoding regions in ES cells may depend predominantly on the activities of Dnmt3a/3b (Fig. 4). Alternatively, the maintenance activities of Dnmt3a/3b may be achieved by the remethylation mechanism of de novo activity in the absence of Dnmt1. The remethylation mechanisms or the proofreading functions (Chen et al. 2003) by Dnmt3a/3b may involve other epigenetic functions such as chromatin remodeling during the course of maintenance of DNA methylation in CpG islands or gene encoding regions. Chromatin configuration affects DNA methylation status and vice versa (Lachner and Jenuwein 2002; Li 2002). To determine the mechanism for maintaining DNA methylation patterns by Dnmt3a/3b, studies on the molecular link between histone modification and DNA methylation will be needed in the future.

The present study demonstrated that Dnmt3a/3b are involved in the maintenance of DNA methylation patterns at CpG islands. Interestingly, T-DMRs with loss of methylation were completely overlapped between *Dnmt1*- and *Dnmt3a/Dnmt3b*-deficient ES cells. Collaboration of Dnmts has been reported in some genomic regions. Cooperation of DNMT1 with DNMT3B for maintenance methylation in a human cancer cell line has been reported (Rhee et al. 2002). Although disruption of either DNMT1 or DNMT3B resulted in a minimal effect on DNA methylation in those cells, double knockout of these genes resulted in marked decrease of DNA methylation in *Igf2*, *p16INK4a*, and repeated sequences (Rhee et al. 2002). In this case, DNMT1 and DNMT3B complement each other in (or share) the maintenance activity of DNA methylation. Involvement of Dnmt3a/3b in maintenance methylation is suggested also by the genetic study at DMR2 region of *Igf2*, the 5′ region of *Xist*, and some repeated sequences (Okano et al. 1999). Dnmt1 and either Dnmt3a or Dnmt3b may cooperate with each other for maintenance of DNA methylation in CpG island in wild-type ES cells, based on the



	wild type	*Dnmt1*$^{-/-}$	*Dnmt3a*$^{-/-}$	*Dnmt3b*$^{-/-}$	*Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$
Repetitive elements					
minor satellite	+	-	+	±	±*
major satellite	+	-	+	+	±*
endogenous retrovirus	+	-	+	+	±*
IAP repeat	+	-	+	+	±*
Imprinted genes					
Igf2r	+	-	+	+	+
H19	+	-	+	+	±
Igf2	+	-	+	+	-
Xist	+	-	+	+	-
CpG islands / Genes					
various loci	+	±	+	+	-

Data are summarized according to the present study and previous reports (Okano et al. 1999; Chen et al. 2003)
* Demethylation is progressive during the course of ES cell culture (Chen et al. 2003).

Figure 4 Summary of DNA methylation at CpG islands and genes, or repetitive elements such as interspersed repeats and centromeric satellites. Although Dnmt1 and Dnmt3a/3b are involved in DNA methylation at both repeated sequences (zigzag lines) and genes (closed boxes) with/without CpG islands (open boxes), Dnmt1 and Dnmt3a/3b act in a different fashion; Dnmt1 functions as a maintenance methyltransferase both in repeated sequences and CpG islands, whereas Dnmt3 primarily functions in CpG islands. *Lower* table represents the methylation statuses at various genomic areas in the wild-type and Dnmt-deficient ES cells reported here or elsewhere (Okano et al. 1999; Chen et al. 2003).

present data that demethylation occurred in CpG islands in both of *Dnmt1*$^{-/-}$ and *Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$ ES cells. It was reported that DNMT1, DNMT3A, and DNMT3B made direct association with each other, and an enhancement of DNA methylation activity was observed when DNMT1 and DNMT3s interacted (Kim et al. 2002). Collectively, Dnmt1 and Dnmt3a/3b may associate directly and function as a complex for DNA methylation in CpG islands. Interaction among the Dnmts and the methyl CpG-binding domain proteins (MBDs), including MeCP2, should also be considered for the mechanism of maintaining DNA methylation patterns by using the proofreading or remethylation function. Furthermore, the mutual dependency on epigenetic memory systems such as DNA methylation and chromatin remodeling should be considered as well.

The present study suggests that Dnmts have preferences for specific genomic regions; Dnmt3a/3b prefer the T-DMR of CpG islands and genes compared with repeated sequences, whereas the nongenic regions including repeated sequences are less dependent on Dnmt3a/3b for maintenance. It is clear that there is some redundancy between the roles of Dnmt3a/3b and Dnmt1 regarding the methylation of gene encoding regions (Fig. 4). No mammalian Dnmts exhibited any sequence specificities in vitro, although there is a report of preferred nucleotide composition around the CpG site for Dnmt3a (Lin et al. 2002). It was demonstrated previously that disruption of Dnmt3a and Dnmt3b resulted in partial demethylation of endogenous C-type retroviruses, L1-like repeats, and major and minor satellites, whereas the lack of Dnmt1 activity caused almost complete demethylation in these sequences (Okano et al. 1999; Liang et al. 2002; Chen et al. 2003). In addition, it was demonstrated that the DMR2 of *Igf2* and 5′ region of *Xist* gene were unmethylated both in *Dnmt1*$^{-/-}$ and *Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$ ES cells, whereas region 2 of *Igf2r* and 5′ upstream region of *H19* were unmethylated in *Dnmt1*$^{-/-}$ ES cells but not or partially demethylated in *Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$ ES cells (Okano et al. 1999). The preference of Dnmts is different depending on the genomic region, providing novel insight into the mechanism of DNA methylation.

METHODS

ES Cells and Genomic DNA Preparation

Mutant ES cell lines deficient of *Dnmt1*$^{-/-}$ (c/c), *Dnmt3a*$^{-/-}$ (6aa), *Dnmt3b*$^{-/-}$ (8bb), and *Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$ (7aabb) were generated in vitro by several rounds of homologous recombination (Li et al. 1992; Lei et al. 1996; Okano et al. 1999), and all ES cells were cultured as previously described (Lei et al. 1996; Okano et al. 1999). Lack of Dnmt protein expression in each mutant ES cell line was previously examined using immunoblotting analyses (Okano et al. 1999; Chen et al. 2003).

Proliferating and undifferentiated ES cells were harvested to isolate genomic DNA as reported previously (Ohgane et al. 1998; Imamura et al. 2001; Shiota et al. 2002). Genomic DNA was extracted when passage numbers of J1, c/c, 6aa, 8bb, and 7aabb were at 11, 36, 16, 19, and 19, respectively.

RLGS and Spot Identification by Cloning and Vi-RLGS

RLGS, which enables us to assess the methylation status of cells or tissues reproducibly (Shiota et al. 2002) was performed according to the previous report (Ohgane et al. 1998; Imamura et al. 2001) using the combination of restriction enzymes, NotI-PvuII-PstI. To block nonspecific labeling, genomic DNA was treated with Klenow fragment (TaKaRa) in the presence of dGTPαS, dCTPαS (Amersham Pharmacia), ddATP, and ddTTP (TaKaRa). DNA was digested with NotI as a landmark enzyme (Nippongene), and the resulting cohesive ends were labeled with Sequenase version 2.0 (USB) in the presence of [α-^{32}P]dCTP and [α-^{32}P]dGTP (Amersham Pharmacia), digested with *Pvu* II (Nippongene), and then subjected to first dimension electrophoresis in a 0.9% agarose disc gel for 23 h at 230 V. After DNA fragments were treated with PstI (Nippongene) in the disk gel, the resulting DNA fragments were separated in second dimensional 5% polyacrylamide gel for 20 h at 150 V. The gel was dried onto chromatography paper (Whatman) and exposed to an Imaging Screen for scanning with Molecular Imager FX (BioRad) or to X-ray film (Kodak XAR5, Eastman Kodak) at −80°C. The spot profiles were analyzed by a 2D/E analyzing system (PDquest, BioRad). For each cell preparation, three to 10 RLGS experiments were performed to evaluate whether RLGS profiles are reproducible.

To clone each RLGS spot, the mixture of radiolabeled and unlabeled samples was applied to RLGS as described above. DNA fragments were electroeluted from the resulting spots, which were punched out in advance, for 20 min at 200 V. Extracted DNA fragments were connected with a NotI adaptor (5′-ACGCCAGGGTTTTCCCAGTCACGACGC-3′ and 5′-p-GGCCGCGTCGTGACTGGGAAAACCCTGGCGT-3′) and PstI adaptor (5′-p-GTGTACTGCACCAGCAAATCC-3′ and 5′-GGATTTGCTGGTGCAGTACACTGCA-3′) followed by removal of excess adaptors with MicroSpin S-400 column (Amersham Pharmacia Biotech) and amplified in two rounds of PCR (30 cycles each) with primers for adaptors (for NotI adaptor: 5′-AGGGTTTTCCCAGTCACGACGCGG-3′, for PstI adaptor: 5′-TTGCTGGTGCAGTACACTGCAG-3′). Amplified fragments were digested with NotI and PstI, ligated into pBluescript II SK- (Stratagene), and then subjected to sequence analysis.

Vi-RLGS software, which processes any sequence data in GenBank or FASTA format and simulates two-dimensional electrophoresis using the resulting fragments, was developed and described elsewhere (Matsuyama et al. 2003). Mouse draft genome sequence (MGCSv3_release3) was downloaded in masked FASTA format from the GenBank ftp site (ftp://ftp.ncbi.nih.gov/genomes/M_musculus/) and processed with the combination of NotI-PvuII-PstI recognition sequences. By matching the Vi-RLGS and "real" RLGS profiles to identify candidate spots, the corresponding sequences were retrieved by clicking the spot on the virtual image and were used as queries for sequence analysis in BLAST (http://www.ncbi.nlm.nih.gov/genome/seq/MmBlast.html) and Ensemble (http://www.ensembl.org/Mus_musculus/) to obtain the surrounding sequence information, chromosomal position, and gene names. By using the sequence information, the primer and probes were designed for methylation sensitive quantitative real-time PCR described below. Identification of repetitive elements in spot data was performed using RepeatMasker (http://ftp.genome.washington.edu/cgi-bin/RepeatMasker).

Sodium Bisulfite Genomic Sequencing

Sodium bisulfite genomic sequencing was performed as previously described (Hattori et al. 2004). Briefly, genomic DNA was digested with PstI and denatured by adding 0.3 M NaOH and incubating for 15 min at 37°C. After the incubation, sodium metabisulfite (pH 5.0) and hydroquinone were added to final concentrations of 2.0 M and 0.5 mM, respectively, and the mixture was further incubated in the dark for 16 h at 55°C. The modified DNA was purified through the Wizard DNA Clean-Up system (Promega), and the bisulfite reaction was terminated with NaOH at final concentration of 0.3 M for 15 min at 37°C. The solution was then neutralized by adding NH_4OAc (pH7.0) to a final concentration of 3 M. The ethanol-precipitated DNA was resuspended in water and then amplified by PCR using primers were designed as follows: spot 152, 5′-GGTGAGTTTTTTTGGATTTAATAAT-3′ and 5′-AACACTAAACACCTAATTTATATCTA-3′; spot 167, 5′-GGTGGTTTTAGAGATTGATATTT-3′ and 5′-CCCTTCATCTACTTCTCTACAA-3′; spot 212, 5′- TTTATAGGAGTTTAGTTGGTTTGTT-3′ and 5′-AAAACAATAATTCTCAACCCATA-3′; and spot 231, 5′-GTTTGATTTTATATTAAAGTATTGG-3′ and 5′-TCACATCAATTTTAACCTCTAAATA-3′. The amplified PCR fragments were cloned into pGEM T-easy vector (Promega) and sequenced for each sample.

Methylation Analysis Based on the Real-Time PCR

Methylation status at specific loci detected by RLGS was evaluated by using the combination of the methylation-sensitive restriction digestion and quantitative real-time PCR (Heid et al. 1996). Genomic DNA was digested by PstI, and the aliquot was treated subsequently with NotI. Each primer set for PCR was designed to amplify the region that included the NotI site detected in RLGS analysis. Forty nanograms of genomic DNA treated with or without NotI were analyzed by real-time PCR with the primers. The amount of undigested DNA both in NotI-treated and -untreated genomic DNA was estimated by real-time PCR with TaqMan Universal PCR Master Mix by using ABI Prism 7000 Sequence Detection System (Applied Biosystems) according to the manufacturer's protocol. The methylation ratio at each RLGS locus was defined as the proportion of the amount of undigested DNA in NotI-treated genome to that in the NotI-untreated one. Initial DNA amount in the reaction mix was normalized with TaqMan Rodent GAPDH Control Reagents VIC Probe (Applied Biosystems). For all samples, at least three independent PCRs performed in duplicate were repeated, Primer and probe sets for quantitative real-time PCR used in this study are as follows: spot 15, 5′-AACAGCTAGGGCAATCCTCTTC-3′, 5′-ACAGTTACAGGTGAGTGTGATCTG-3′, and 5′-Fam-CCGCCTGCTGATGCTCATGGCTCT-Tamra-3′; spot 34, 5′-CAGGTACACCACATGAGGTCTC-3′, 5′-CTCCCAAGGTCGTTCTAAAGAGTA-3′, and 5′-Fam-ACACAGCCATCTGGTGCTTTGCGT-Tamra-3′; spot 115, 5′-GATGTACCTGGCCCTCAACTG-3′, 5′-AGTCTGCCGAAGTCCTGTGA-3′ and 5′-Fam-CCAGCCCACCTTACCCCAAGCCCA-Tamra-3′; spot 152, 5′-GTCTTAGCTCCGTTTCTTCTTTCG-3′, 5′-GGGCACATAGCAATAACTGGGT-3′, and 5′-Fam-CCAGCAGCTCGCAACGTGAACGGT-Tamra-3′; spot 167, 5′-GATCTGGAGTCAGTCTCTTCACT-3′, 5′-GTGAACATCTTTACTCCATGACCA-3′, and 5′-Fam-CAAACCCGAGTGTCTCCTGCCTGG-Tamra-3′; spot 169, 5′-CCTCCGGCAGATGAATGCTAA-3′, 5′-CACACTGGCTGACCTGGATC-3′, and 5′-Fam-ACCCGCCTCTCCTTAGTGTGCCCTTamra-3′; spot 178, 5′-GCCCTCCAGTAAAAGTGAAGC-3′, 5′-TGATCTCTGACCTCTGCACATATA-3′, and 5′-Fam-AGCTGCACAATGACCCCTCCTGAC-Tamra-3′; spot 212, 5′-GTAATTCTGGCTTGGGCTATAAGA-3′, 5′-ACAGCCGCAAAATTACAATTAACA-3′, and 5′-Fam-TTCCCTGTTGCTGCTACTGCTGGT-Tamra-3′; and spot 231, 5′-GCTTTCACATCTAAGAGACTGACC-3′, 5′-CGTTCTTGGATGGGCTGTGT-3′, and 5′-Fam-CCAGCAGCGTGTGCGCGGCC-Tamra-3′.

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